bcl-2 Oncoprotein in Surgically Resected Nonsmall Cell Lung Cancer: Possibly Favorable Prognostic Factor in Association With Low Incidence of Distant Metastasis

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Background: The *bcl-2* oncoprotein serves a regulatory function in permitting several cell types to die in an apoptotic process. Its overexpression probably plays a role in tumorigenesis and tumor development. The aim of this study was to determine the clinicopathological and prognostic significance of the *bcl-2* oncoprotein in patients with nonsmall cell lung cancer (NSCLC).
Methods: Immunostaining for *bcl-2* oncoprotein was performed on 182 operable NSCLCs.
Results: Thirty-six patients (19.8%) showed a positive immunostaining for *bcl-2* oncoprotein. Histologically, its incidence was higher in squamous cell carcinomas (29.6%). Its expression status was inversely correlated with tumor development-associated parameters such as tumor stage in NSCLCs, especially in squamous cell carcinomas. *bcl-2* positive patients with NSCLCs, especially squamous cell carcinomas, showed better overall survival and disease-free survival (DFS). In a multivariate analysis, this oncoprotein status had prognostic value in DFS for NSCLCs and in overall survival for squamous cell carcinomas. The recurrence of *bcl-2* positive NSCLCs was significantly uncommon in distant extrathoracic organs.
Conclusions: The expression of *bcl-2* oncoprotein in NSCLCs may be an early event of tumor development, especially in squamous cell carcinomas, and may be of importance in determining tumor progression and prognosis.
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KEY WORDS: **nonsmall cell lung cancer; *bcl-2* oncoprotein; prognosis; recurrence; distant metastasis; immunohistochemistry**

INTRODUCTION

The *bcl-2* oncoprotein has been reported to be encoded by a gene activated as a consequence of the t(14;18) chromosomal translocation in human follicular lymphomas [1]. This abnormality brings the *bcl-2* gene into juxtaposition with the immunoglobulin heavy chain locus and subsequently causes overexpression of the *bcl-2* oncoprotein [2,3]. The biological function of its overexpression remains uncertain, but recent evidence suggests that this oncoprotein pathophysiologically serves a regulatory function in permitting several cell types to die in an apoptotic process. Avoidance of induced or spontaneous apoptosis via *bcl-2* oncoprotein may thus result in a survival advantage [4]. Apart from cells of the lymphoid

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lineage, *bcl-2* oncoprotein overexpression has been observed in a wide range of normal epithelia [5], and the *bcl-2* oncoprotein has recently been demonstrated to be abnormally expressed in several precancerous or cancerous lesions [6–22]. In contrast to lymphomas, gross alterations in the *bcl-2* gene structure are not observed, indicating that alternative mechanisms of deregulation of *bcl-2* oncoprotein expression may exist in such lesions [23].

Whereas small cell lung cancer (SCLS) commonly expresses *bcl-2* oncoprotein [24–27], nonsmall cell lung cancer (NSCLC) expresses it at a relatively low incidence of 20–67% [26, 28–32]. However, the clinicopathological significance of this oncoprotein expression in lung cancer remains controversial. Fontanini et al. [32] suggested that the *bcl-2* oncoprotein status is a favorable prognostic factor in NSCLCs, whereas Pezzella et al. [28] found it useful only in squamous cell carcinoma, and Ritter et al. [31] only in well-differentiated squamous cell carcinoma at an early stage. This suggests that *bcl-2* oncoprotein is associated with tumor progression and prognosis in some limited subsets of lung cancer, whereas Gaffney et al. [29] did not find such an association. However, in these previously reported studies, there are apparently several problems: the relatively small sample examined [29], differences in criteria of positivity for *bcl-2* oncoprotein expression; histological imbalance of the sample ratio between squamous cell carcinoma type and adenocarcinoma type [28,32]; and undetermined difference of metastatic pattern. These problems indicate a pressing need for further analyses of clinicopathological and prognostic implications of *bcl-2* oncoprotein in lung cancer, especially in NSCLCs.

Herein, we examined the clinicopathological and prognostic significance of *bcl-2* oncoprotein expression in NSCLCs, by analyzing immunohistochemically *bcl-2* oncoprotein expression of surgically resected materials using anti-*bcl-2* oncoprotein monoclonal antibody and especially by correlating its expression with prognosis, as well as the pattern of tumor recurrence.

MATERIALS AND METHODS

Patients

Of a total of 299 patients who had undergone surgery for NSCLC between July 1989 and June 1993 at our institution, 182 patients, whose materials were well preserved in a frozen condition for immunohistochemistry, were studied. The patients (135 men and 47 women, mean age 64.2, range 35–82) were followed up for a median time of 34.6 months (range 1.1–75.0 months). According to the international TNM staging system [33], 106 were in pathological stage I (p-stage I), 17 were in pathological stage II (p-stage II), 45 were in pathological stage IIIA (p-stage IIIA), and 14 were in pathological stage IIIB (p-stage IIIB). The patients' histological type was 71 squamous cell carcinomas, 97 adenocarcinomas, 12 large cell carcinomas, and 2 adenosquamous cell carcinomas. Fifty-two were histologically well differentiated, 82 were moderately differentiated, and 48 were poorly differentiated. The patients in the present series underwent no preoperative adjuvant therapy, and potentially curative surgery was performed on 174 patients.

Immunostaining

Immunostaining was performed on cryostat sections with an avidin-biotin peroxidase complex method as previously described [8,26,28] with the use of a monoclonal antibody specific for *bcl-2* oncoprotein (clone *bcl-2*-124, dilution 1:50, Dako, Glostrup, Denmark). Staining without this antibody was routinely performed as a negative control procedure, and positive staining of some small lymphocytes in the sections provided an internal control for *bcl-2* staining.

The *bcl-2* oncoprotein immunoreactivity was assessed by five high-power fields ($\times$40, objective lens), and the mean number of *bcl-2*-positive tumor cells was counted: patients with no or less than 10% positive cells within the tumor tissue were judged as *bcl-2* negative, and those with more than 10% as *bcl-2* positive (Fig. 1).

Statistical Analysis

The χ^2 test was applied for statistical analysis. The patients' survival data were used to determine the possible correlation between *bcl-2* oncoprotein expression and cancer-associated death, and survival curves were constructed by the Kaplan-Meier method. The statistical significance of these data was analyzed by the log-rank test. Variables influencing survival were analyzed by Cox's proportional hazards regression model with SAS software (Statistical Analysis System Institute, Cary, NC). A P value of <0.05 was considered statistically significant, and a P value of <0.10 was considered marginally significant.

RESULTS

bcl-2 Oncoprotein Expression and Clinicopathological Factors

Thirty-six patients (19.8%) in this series showed positive immunoreactivity for *bcl-2* oncoprotein. Table I shows a summary of the association between *bcl-2* oncoprotein expression and representative clinicopathological factors in NSCLC. The incidence of *bcl-2* positivity was 29.6% (21/71 patients) in squamous cell carcinoma, 14.4% (14/97 patients) in adenocarcinoma, 8% (1/12 patients) in large cell carcinoma, and 0% (0/2 patients) in adenosquamous cell carcinoma, respectively. Since the incidence of *bcl-2* oncoprotein positivity in squamous cell carcinoma was significantly higher ($P = 0.014$) than that in nonsquamous cell carcinoma, Table II summarizes the relationship between its expression and clinicopatho-

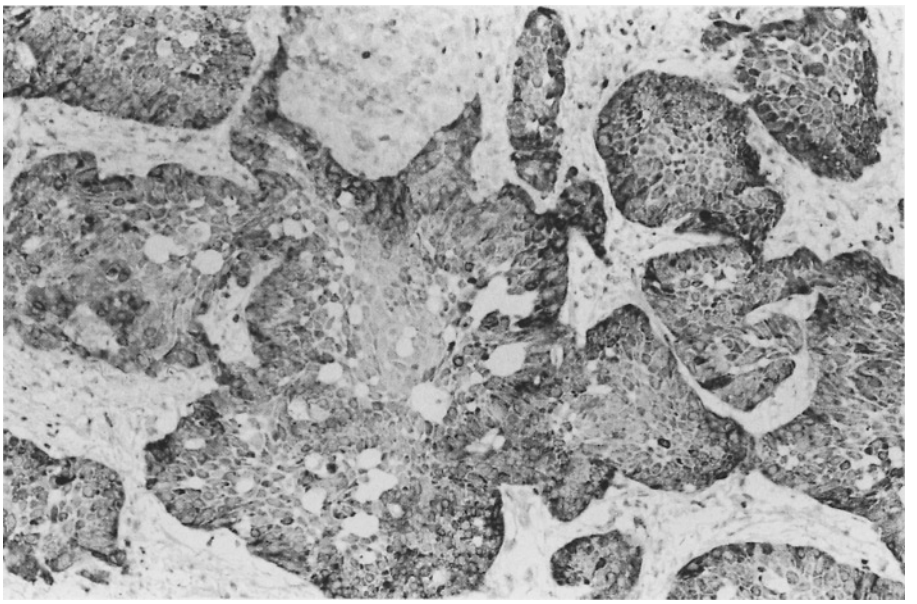

Fig. 1. Immunostaining for *bcl-2* oncoprotein expression in NSCLCs. *bcl-2* oncoprotein is expressed in a majority of tumor cells within squamous cell carcinoma tissue.

logical factors, separately for squamous cell carcinoma and non-squamous cell carcinoma. Overall in NSCLCs, *bcl-2*-positive patients showed a significantly earlier stage of disease than *bcl-2*-negative patients ($P = 0.012$), in relation to T classification ($P = 0.054$). In squamous cell carcinoma, *bcl-2* oncoprotein expression was significantly more frequently observed ($P = 0.002$) in early stage patients, especially in relation to T classification ($P = 0.071$) and N-classification ($P = 0.028$). By contrast, there was no significant association betweem *bcl-2* oncoprotein expression and clinicopathological factors in patients with nonsquamous cell carcinoma.

Survival Analysis

For 174 patients with NSCLC undergoing potentially curative surgery, postoperative overall survival and disease-free survival (DFS) were analyzed according to *bcl-2* oncoprotein expression status (Fig. 2A,B). The patients with *bcl-2* positive NSCLC showed significantly better prognosis than those with *bcl-2* negative NSCLC (overall survival: $P = 0.039$, DFS: $P = 0.031$). Among 107 patients with nonsquamous cell carcinoma, *bcl-2* oncoprotein expression status had no influence on overall survival ($P = 0.427$) or disease-free survival ($P = 0.419$) (data not shown), but among 67 patients with squamous cell carcinoma, *bcl-2* positive patients showed signficantly more favorable prognosis than *bcl-2* negative patients (overall survival: $P = 0.030$, DFS: $P = 0.042$) (Fig. 2C,D). Even among stage I patients with squamous cell carcinoma, *bcl-2* positive patients showed a favorable prognosis of marginal significance in overall survival (Fig. 2E, $P = 0.054$).

TABLE I. Relationship Between *bcl-2* Oncoprotein Expression and Clinicopathological Factors in 182 Patients With Nonsmall Cell Lung Cancer

	bcl-2 negative n = 146	*bcl-2* positive n = 36	*P* value
Sex male/female	107/39	28/8	0.735
Age mean ± SD (years)	63.7 ± 8.8	66.1 ± 8.9	0.170
Histology[a] Sq	50	21	
Ad	83	14	
La	11	1	
As	2	0	*0.014*[b]
Stage I	80	26	
II	11	6	
IIIA	41	4	
IIIB	14	0	*0.012*
T classification			
T1	49	13	
T2	66	23	
T3	20	1	
T4	11	0	*0.054*
N classification			
N0	92	26	
N1	15	6	
N2,3	39	4	0.112
Tumor size (mm)			
<31	60	14	
31 ≤ <61	71	19	
61 ≤	15	3	0.885
Histological differentiation			
Well	38	14	
Moderate	65	17	
Poor	43	5	0.112

[a]Sq = squamous cell carcinoma, Ad = adenocarcinoma, La = large cell carcinoma, As = adenosquamous cell carcinoma.
[b]Sq vs. nonsquamous cell carcinoma.
Italics = statistically significant or marginally significant.

TABLE II. Relationship Between *bcl-2* Oncoprotein Expression and Clinicopathological Factors in 71 Patients With Squamous Cell Carcinoma and 111 Patients With Nonsquamous Cell Carcinoma

A: Squamous cell carcinoma (n = 71)			
	bcl-2 negative n = 50	*bcl-2* positive n = 21	*P* value
Stage I	23	15	
II	1	4	
IIIA	17	2	
IIIB	9	0	*0.002*
T classification			
T1	7	6	
T2	27	14	
T3	7	1	
T4	9	0	*0.071*
N classification			
N0	28	15	
N1	3	4	
N2,3	19	2	*0.028*
B: Nonsquamous cell carcinoma (n = 111)			
	bcl-2 negative n = 96	*bcl-2* positive n = 15	*P* value
Stage I	57	11	
II	10	2	
IIIA	24	2	
IIIB	5	0	0.562
T classification			
T1	42	7	
T2	39	8	
T3	13	0	
T4	2	0	0.413
N classification			
N0	64	11	
N1	12	2	
N2,3	20	2	0.794

Italics = statistically significant or marginally significant.

Multivariate analyses using Cox's proportional hazards model for patients with NSCLC and squamous cell carcinoma are summarized in Table III. *bcl-2* oncoprotein expression was a marginally independent favorable prognostic factor influencing DFS among NSCLC patients ($P = 0.092$) and also on overall survival among patients with squamous cell carcinoma ($P = 0.081$).

Recurrent Sites Analysis

Table IV shows the relationship between *bcl-2* oncoprotein expression status and postoperative initial recurrent sites in potentially curatively resected patients with NSCLC at the time of November 20, 1995. Among *bcl-2* positive patients, the incidence of recurrence in distant extrathoracic organs such as bone, brain, liver, adrenal glands, and kidneys was low (6%, $P = 0.011$), in comparison with *bcl-2* negative patients (25%). This finding was obtained when separately analyzing for the patients with squamous cell carcinoma and nonsquamous cell carcinoma (data not shown). In contrast, there was no difference in the incidence of recurrence locoregionally, the lungs, or the extrathoracic lymph nodes.

DISCUSSION

The clinicopathological and prognostic significance of *bcl-2* oncoprotein expression in human malignancies remains controversial. Even in non-Hodgkin lymphoma, including follicular type with the t(14;18) chromosomal translocation, *bcl-2* oncoprotein expression status has been reported to be associated with high grade malignancy [34], or low grade malignancy [35], or to be unassociated with malignant grade [36, 37]. In breast cancer, in which *bcl-2* oncoprotein correlates with well-differentiated carcinoma, hormone receptor expression, and sensitivity to endocrine therapy, its expression is now considered to be associated with favorable clinicopathological features [6–11], although Sierra et al. [12] described the apparently conflicting data in the well-differentiated subtype of breast cancer. In colorectal cancer, Bosari et al. [16] found a lack of prognostic significance, yet Ofner et al. [17] recently described that *bcl-2* oncoprotein was strongly associated with a favorable clinical outcome. In thyroid cancer, *bcl-2* oncoprotein was more frequently expressed in the well-differentiated type, and the possibility was raised that its expression may be a favorable prognostic factor linked to differentiation [13]. By contrast, in prostatic cancer, *bcl-2* oncoprotein expression may be an indicator of high grade malignancy in association with neuroendocrine differentiation [21] as well as resistance to hormone therapy [19,20]. In neuroblastoma [18], its expression was strongly liked to N-myc amplification and to a poor prognosis. However, there was no association between its expression and malignant grade in some other tumors [14,22]. Thus the role of *bcl-2* oncoprotein expression in tumor malignancy seems to differ with the type of tumor, depending on various biological characteristics such as histological type, histological differentiation, neuroendocrine differentiation, hormone receptor status, and status of oncogene abnormalities such as p53, *erbB*-1, *erbB*-2, and N-*myc*.

Small cell lung cancer frequently expresses *bcl-2* oncoprotein [24–27], but the incidence of its expression in NSCLCs is significantly low [26,28–32]. However, even among NSCLC patients, it is noteworthy that those with squamous cell carcinoma also showed a higher incidence of *bcl-2* oncoprotein expression than those with other histological types. Similar findings were obtained in other studies [28,30–32]. Therefore, when estimating the clinicopathological implications of this protein expression in lung cancer, histological classification into three groups, i.e., small cell lung cancer, squamous cell carcinoma and others, mainly adenocarcinoma, may be needed. Indeed, in the present study, *bcl-2* oncoprotein expression in NSCLCs appeared to be inversely associated with tumor

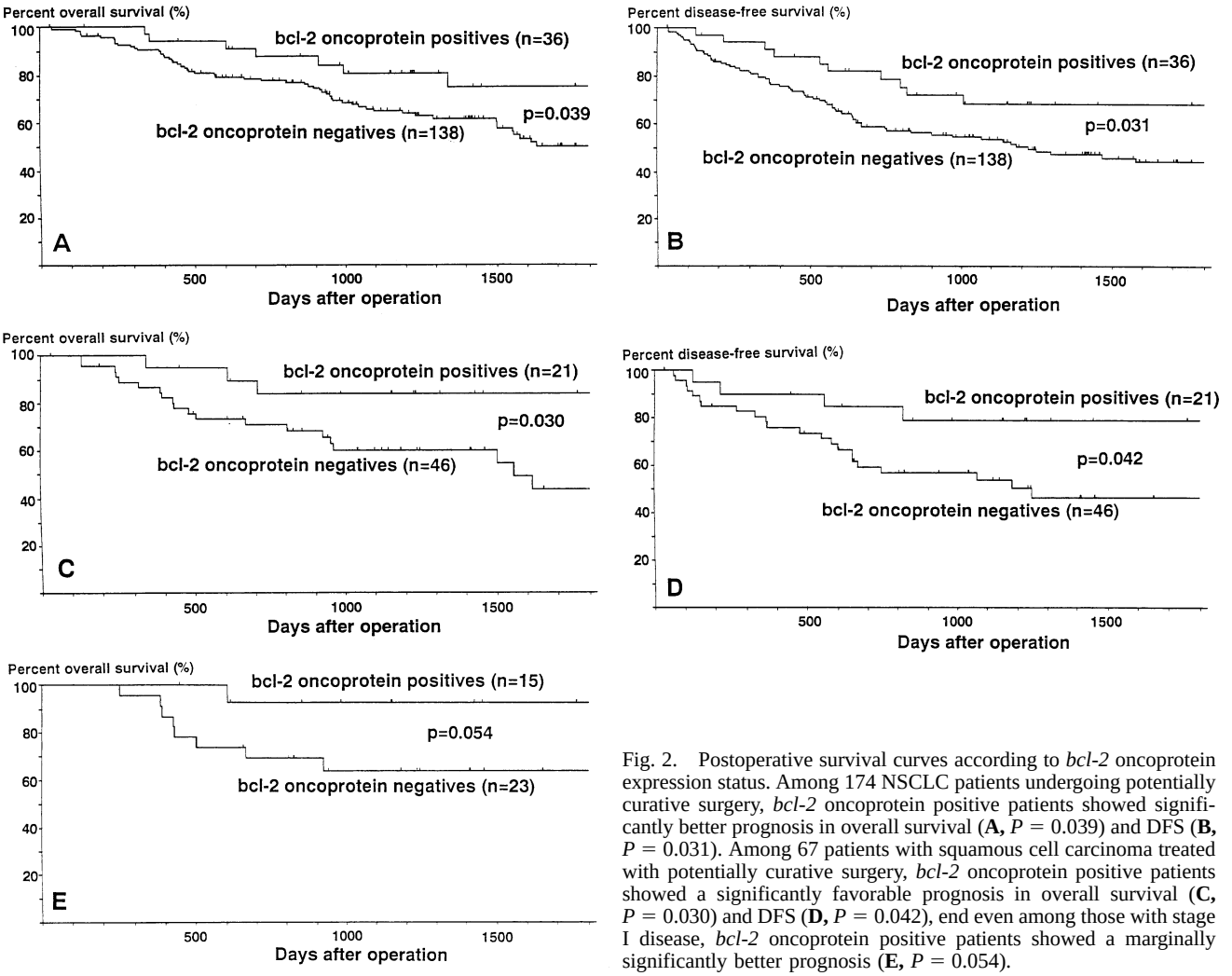

Fig. 2. Postoperative survival curves according to *bcl-2* oncoprotein expression status. Among 174 NSCLC patients undergoing potentially curative surgery, *bcl-2* oncoprotein positive patients showed significantly better prognosis in overall survival (**A,** $P = 0.039$) and DFS (**B,** $P = 0.031$). Among 67 patients with squamous cell carcinoma treated with potentially curative surgery, *bcl-2* oncoprotein positive patients showed a significantly favorable prognosis in overall survival (**C,** $P = 0.030$) and DFS (**D,** $P = 0.042$), end even among those with stage I disease, *bcl-2* oncoprotein positive patients showed a marginally significantly better prognosis (**E,** $P = 0.054$).

TABLE III. Multivariate Analysis of Cox's Proportional Hazards Model in Patients Undergoing Potentially Curative Surgery

	Multivariate analysis (*P*-value)			
	NSCLC[a] (n = 174)		Sq[b] (n = 67)	
Variable	Overall[c]	DFS[d]	Overall[c]	DFS[d]
Tumor size (mm) <35/35≤	0.155	0.581	0.701	0.300
T classification T1,2/T3,4	0.257	0.415	0.726	0.806
N classification N0/N1-3	*0.0003*	*0.00002*	*0.014*	*0.074*
Differentiation Well, moderate/poor	0.102	*0.047*	0.490	0.433
bcl-2 oncoprotein Positive/negative	0.159	*0.092*	*0.081*	0.196

[a] Nonsmall cell lung cancer.
[b] Squamous cell carcinoma.
[c] Overall survival.
[d] Disease-free survival.
Italics = statistically significant or marginally significant.

TABLE IV. Relationship Between *bcl-2* Oncoprotein Expression Status and Recurrent Sites in 174 Nonsmall Cell Lung Cancer Patients Undergoing Potentially Curative Surgery

	bcl-2 negative n = 138 (%)	*bcl-2* positive n = 36 (%)
Disease-free (n = 95)	69 (50)	26 (72)
Locoregional (n = 11)	9 (7)	2 (6)
Lung (n = 16)	13 (9)	3 (8)
Distant extrathoracic metastasis (n = 52)		
lymph node (n = 16)	13 (9)	3 (8)
Others (n = 36)[a]	34 (25)	2 (6)[b]

[a] Bone: n = 9, liver: n = 6, brain: n = 15, others: n = 6.
[b] Adenocarcinoma, bone metastasis, n = 1; squamous cell carcinoma, kidney metastasis, n = 1; *bcl-2* positive vs. *bcl-2* negative, $P = 0.011$.

development-associated factors and led to better prognosis, but these findings were mainly obtained in squamous cell carcinoma, not in nonsquamous cell carcinoma. Therefore, the ratio of squamous cell carcinoma to nonsquamous cell carcinoma may be an important factor in determining its implications overall in NSCLCs. In our

series, its ratio was 71 to 111 patients, being a reasonable number for surgically resected NSCLCs, whereas several other studies were performed using large numbers of patients with squamous cell carcinoma [28,32].

In our study, patients with *bcl-2* positive squamous cell carcinoma were included in earlier stage, T-classification, and N-classification and showed favorable prognosis. In spite of the small number of the examined materials, even stage I patients with squamous cell carcinoma showed a similar prognosis. In a multivariate analysis, *bcl-2* oncoprotein status was a marginally independent prognostic factor influencing survival. These findings were obtained, irrespective of patient age (data not shown), unlike those by Pezzella et al. [28].

Moreover, according to analysis of recurrent pattern in patients with NSCLC, the incidence of metastasis in extrathoracic distant organs such as brain, liver, and bone was significantly lower (6%) in *bcl-2* positive NSCLCs than that (25%) in *bcl-2*-negative NSCLCs ($P = 0.011$), suggesting that *bcl-2* positive NSCLCs might rarely show distant hematogenous metastasis. However, considering that recurrence not only in locoregional lymph nodes but also in extrathoracic distant lymph nodes was observed irrespectively of *bcl-2* oncoprotein expression status, *bcl-2* oncoprotein expression did not seem to be associated with the metastatic pathway via lymphatic vessels as a whole. Thus, especially among patients with squamous cell carcinoma, *bcl-2* oncoprotein expression is a possibly early event of tumor development and may be of biological importance in determining tumor progression and prognosis.

The mechanism underlying the effect of *bcl-2* oncoprotein expression on tumor progression and prognosis remains essentially uncertain. Originally, *bcl-2* oncoprotein possesses a function to escape from apoptotic death, resulting in longer cellular survival [4]. In fact, the observations of this oncoprotein distribution in normal tissues and embryonal tissues indicate that its oncoprotein function is displayed in morphogenesis linked to cell proliferation via escape from cell death [4,5,38], but in NSCLCs, Fontanini et al. [32] described that *bcl-2* oncoprotein expression status was at least not correlated with proliferative potential indicators including PCNA and Ki-67. Although *bcl-2* oncoprotein expression in squamous cell carcinoma of the lung may be an early event of tumor development, considering the rarity of distant extrathoracic metastasis in NSCLCs expressing this oncoprotein, we rather emphasize that *bcl-2* oncoprotein plays an inhibitory role in the hematogenous metastatic process through tumor progression. The question of whether *bcl-2* oncoprotein biologically participates in the hematogenous metastatic process and reduces the incidence of distant metastasis may be elucidated in the future.

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